UNIVERSITY OF TASMANIA

Kinetic Performance Potential of Different Column Formats and Separation Modes for Liquid Chromatography

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Declaration

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Tim Causon

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Statement of Co-Authorship

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Abstract

This thesis deals with theoretical and experimental studies on optimisation of separation systems performed using liquid chromatography. The two major themes of work were improving the quality of separations by maximising efficiency with respect to the analysis time (kinetic performance) and by using temperature as an experimental variable. These themes were explored for a range of separation modes and column formats utilised in liquid chromatography.

Included in this thesis is a review of the principles and practices of kinetic performance optimisation for liquid chromatography. Detailed concepts covered in this thesis are introduced and future directions for chromatographic supports discussed.

The role of elevated temperatures (up to 160°C) for practical and performance benefits for organic polymer monolithic stationary phases is examined for typical biomolecule applications. Temperature variation, particularly rapid temperature pulsing, during the separation process is examined using ion-exchange and hydrophilic interaction liquid chromatography modes with capillary columns and a resistively heated column module.

The current performance limitations for the analysis of small anions by ion-exchange chromatography are explored. Additional *in silico* data are used to transform experimental isocratic data to gradient performance predictions for any normalised ramp rate and physicochemical conditions. Examples of high efficiency (25,000-40,000 theoretical plates) and sample peak capacity (n_c = 84) were realised and closely matched predicted values.

A theoretical study of the open tubular column format for liquid chromatography with a thick, porous layer of stationary phase shows the potential of this column format to

improve chromatographic performance with a trade-off for mass loadability. It can be shown that total column diameters should be less than 6 μm with column lengths typically greater than 0.8 m for *N* values in the range of 125,000–500,000 at a maximum pressure of 400 bar. Elevated temperature LC (90°C) is also shown to increase the allowable total column diameter to up to 9 μm for a larger range of *N* values (100,000–880,000).

Introduction

1. Background

The term 'chromatography' is used to describe a broad collective of techniques applied to the separation of mixtures. In classical chromatography two primary phases exist: a flowing *mobile phase* for transportation through an immobilised *stationary phase* support. A mixture of compounds may be introduced to the flowing mobile phase and individual compounds can experience differential migration in this system due to differences in their affinity for each phase (*i.e*. according to their partition coefficients) leading to separation. Chromatography is a particularly valuable technique as it allows exploitation of the chemical properties of compounds to effect a separation for preparative or analytical purposes.

Currently, the ability of many chromatographic techniques to provide adequate separation is exceeded by the complexity of samples requiring analysis. As the fundamental processes generally remain the same for the wide range of chromatographic techniques that exist, the quality (or performance) of any chromatographic analysis can be subject to broad optimisation strategies based on theoretical knowledge and experiment.

2. Fundamentals of liquid chromatography

2.1. Liquid-solid chromatography

The cornerstone of chromatography is the bulk differences in migration experienced by compounds resulting in separation [1]. While the dynamics of this process at a molecular level are chaotic as each molecule interacts with the stationary and mobile phases independently of the others, compounds are observed at a macroscopic level to migrate smoothly as zones at velocities less than or equal to the bulk mobile phase velocity (u_0).

This differential migration exhibited by compounds is determined by the partitioning ratios between the mobile and stationary phases. A retention time (t_R) is recorded and u_0 determined by the residence time of an unretained marker (t_0) with respect to the length of the stationary phase bed. This is frequently quantified for a given compound as the retention factor (k):

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

In the case of *liquid-solid chromatography*, the *liquid* mobile phase passes through the *solid* stationary phase; this is commonly shortened to *liquid chromatography* (LC). The dynamics of LC are somewhat different than for gas phase separations (gas chromatography) as the diffusion coefficients and compressibility of the mobile phase are substantially lower while the viscosity of this phase is much greater. In the case of high pressure liquid chromatography (HPLC), movement of the mobile phase is produced *via* pressurised pumping of the mobile phase through the stationary phase bed. The stationary phase for HPLC is typically a packed bed of small spherical particles (1-100 μm in diameter) or some form of continuous, permeable structure. Compounds (solutes) are dissolved in a liquid medium and introduced as a narrow band into the flowing stream of mobile phase pumped through the stationary phase bed typically contained in a cylindrical column. Solutes that migrate (elute) from the column are collected and/or detected by an analytical device to allow identification and/or quantification of these zones (peaks).

2.2. High pressure liquid chromatography in practice

The principal requirements of any HPLC system are a pump for delivering mobile phase at a constant volumetric flow rate or pressure, an injector for sample introduction, a column

containing a bed of stationary phase and a detector for quantification and/or identification of eluted solutes recorded as a chromatogram.

Pumps used for HPLC must be capable of delivering accurate and reproducible volumetric flow rates with minimal pulsations. The pumping pressures utilised for HPLC are typically less than 400 bar, but recent development in column technology including sub-2 μm particles has necessitated the development of very high pressure pumps capable of exceeding 1000 bar to generate sufficiently high mobile phase velocities. It is also of note that the majority of HPLC pumps are designed to operate in constant flow mode, but it is also possible to operate under conditions of constant pressure. Many HPLC pumps are also capable of mixing and accurately proportioning two or more sources of eluent for the purpose of varying the mobile phase composition during an analysis (*i.e*. gradient elution).

The injector for HPLC is simply a device employed to accurately introduce a known volume of sample into the moving stream of mobile phase close to the head of the column. Most common instruments utilise two-position valves to divert flow of the mobile phase through a loop containing a set volume of sample.

Columns used for HPLC are highly varied in their length, diameter, type of stationary phase material and chemical functionality. In general the column used must be compatible with the mobile phase employed, have some affinity for the solutes of interest and be sufficiently robust to survive operation under the physicochemical conditions employed. A more detailed discussion of column design is contained in Section 4.

Finally, a detector is required for the analytical purpose of chromatography – that is to quantify and (if possible) identify the solutes in the sample mixture. Detectors may be based on a wide range of detection principles including differences in refractive indices, spectroscopic properties (*e.g*. ultraviolet wavelength absorbance, fluorescence), aerosol

based (*e.g.* evaporative light scattering, mass spectrometry) or utilising a post-column reaction of eluted compounds (*e.g.* electrochemical, chemiluminescence).

3. Performance characterisation and optimisation

3.1. Theoretical plate concept

Eluted solutes detected post-separation typically yield a peak exhibiting an approximately normal (Gaussian) distribution with a standard deviation that is governed by band broadening processes. A convenient method to quantify the amount of band broadening can be found in plate theory, which has its roots in describing distillation processes [2]. While the theoretical validity of this approach is not in agreement with the molecular dynamics taking place, it provides a convenient and accurate method to yield a numerical value for one quality of the separation [1].

In the case of a single chromatographic peak, a relationship between the width of the peak (w) and the retention time (t_R) describes the "efficiency" of the peak. The value of the theoretical plate number (N) can be determined using the retention time at the peak maximum (t_R) and the standard deviation of the population (σ). The standard deviation is estimated by assuming a normal (Gaussian) distribution of the population of solute molecules and can be calculated using Eq. 2 with the corresponding peak width values shown in Figure 1.

$$N = \left(\frac{t_R}{\sigma}\right)^2 = m\left(\frac{t_R}{w}\right)^2 = 5.54\left(\frac{t_R}{w_{1/2}}\right)^2 = 16\left(\frac{t_R}{w_{4\sigma}}\right)^2 \quad (2)$$

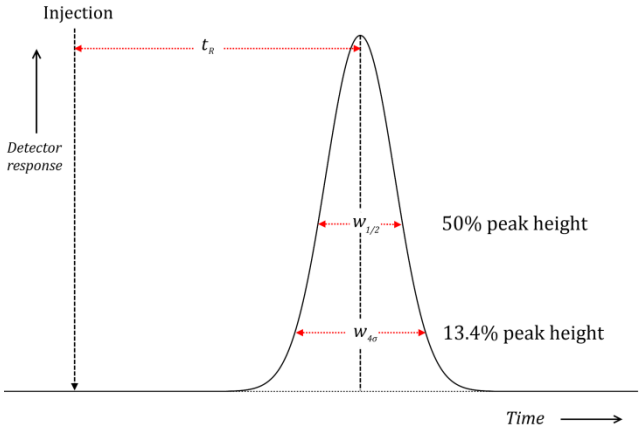

Figure 1. Simplified representation of a chromatogram showing parameters for calculation of the theoretical plate number (*N*).

As separations are performed on columns of a fixed length, the theoretical plate number (*N*) and the column length (*L*) are related through the height equivalent to one theoretical plate ($H=L/N$). The fundamental work of van Deemter [3] provides the framework for describing sources of band broadening during the chromatographic process. The relationship between *H* and mobile-phase velocity (u_0) is described as the sum of different band-broadening contributions:

$$H = A(d_p) + \frac{B}{u_0} + C(d_p^2)u_0 \qquad (3)$$

where the *A*-term is the eddy-diffusion term describing dispersion due to differences in magnitude and direction of the flow through a chromatographic column. The *B*-term describes the longitudinal-diffusion contribution to total band broadening due to Brownian motion. The *C*-term represents the lack of mass equilibrium in the mobile phase (C_m) and that in the stationary phase (C_s).

Clearly, the *A* and *C* terms in Eq. 3 can be improved by reducing the particle diameter yielding a lower *H* value at a given linear velocity. This is illustrated in Figure 2, where plate height values for some typically used particle diameters (d_p = 2, 3 and 5 µm) are shown for a range of linear velocities. This type of representation is often described as a "van Deemter plot". It is further possible to define analogous reduced terms (*i.e.* dimensionless parameters) to allow comparison of columns packed with different particle sizes or column diameters [1].

This type of analysis provides the basis for optimising the theoretical plate number by selection of the optimum mobile phase velocity (*i.e.* the minimum of the *H*, u_0 curve). However, to compare performance with some consideration to the available time for analysis (*i.e.* plates/time) the maximum available pressure drop (ΔP_{max}), the column length and the physicochemical conditions must also be taken into account.

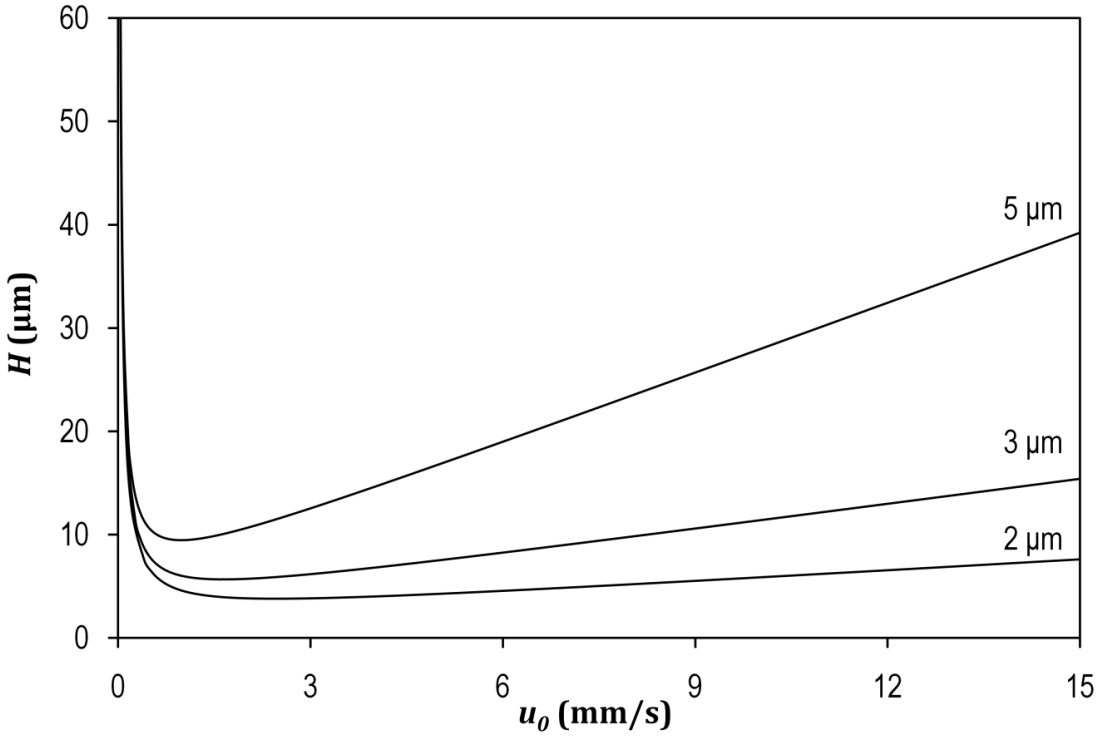

Figure 2. *H*, u_0 curves calculated using Eq. 3 for typical parameters (*A* = 1, *B* = 2, *C* = 0.1) and d_p values of 2, 3 and 5 µm.

3.2. Kinetic performance

One possibility for performance characterisation not considered by general plate height equations is the increase in pressure drop with the inverse square of the particle size which limits the maximum column length or mobile phase velocity that can be employed; this affects the maximum plate number that can be generated or the minimum required analysis time.

To address this trade off between N and time, a graphical approach to compare the kinetic performance in terms of efficiency and analysis time of separations that differ in nature (*i.e*. HPLC versus gas chromatography) was introduced by Giddings in 1965 [4]. This type of plot provides an indication of the time required to obtain a specific number of plates at a given maximum pressure drop for columns of different length. This work was continued by Poppe who introduced a method using iterative calculations to generate a plot of the pressure-drop limited plate number as a function of N/t_0 [5]. More recently, Desmet *et al*. [6] introduced a series of kinetic plots derived from experimental H, u_0 and column permeability data for unbiased comparison of different stationary phase formats and chromatographic conditions. A detailed explanation of this type of performance characterisation is given in **Chapter I**.

3.3. Chromatographic resolution

Beyond optimisation of the plate height for the analysis of a single solute, assessment of the quality of any chromatographic analysis requires a quantitative measure of how well two or more peaks of interest are separated. Plainly speaking, for any two peaks of interest we can consider the width of the peaks, their retention times and the ratio of their respective retention factors (α). The overall quality of a separation between two peaks is conventionally calculated using the resolution factor (R_s). Eq. 4 shows that resolution is

influenced by three important factors: the theoretical plate number (N), the retention factor (k'), and the selectivity (α).

$$R_s = \frac{2(t_{R,2}-t_{R,1})}{w_1+w_2} = \frac{\sqrt{N}}{4}.\frac{k}{k+1}.\frac{\alpha-1}{\alpha+1} \quad (4)$$

Typically the selectivity (mobile and stationary phase chemistry) is optimised prior to the theoretical plate number and kinetic performance. This involves selection of a mobile and stationary phase combination with suitable properties to effect retention and elution of the compounds of interest. After these optimisations, there also remain possibilities for improving the resolution by variation of the mobile phase during the analysis by direct means (*e.g.* gradient HPLC) or by indirect means (*e.g.* temperature variation). However, these are not exclusive effects and experimental investigation often yields more complex results than predicted by theory [7]. Thus there is a wide range of possibilities for optimising both N and R_s in chromatographic method development.

3.4. Influence of temperature

One variable that can be used to manipulate chromatographic resolution by changing efficiency and retention characteristics is the temperature at which the analysis is performed. Thermodynamically, the retention of components in HPLC can be described by a form of the van't Hoff equation:

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln\beta \quad (5)$$

where T is the temperature, R is the universal gas constant, ΔH and ΔS are the partial molar change in enthalpy and entropy (respectively) of transfer from the mobile to

stationary phase, and β is the phase ratio (*i.e*. the ratio of volume of mobile phase to that of stationary phase).

From this equation, it can be seen that increasing temperature should result in a predictable decrease in retention. However, deviations from linearity derived from particular functional groups within a solute (*e.g*. ionisable groups), temperature related changes in the mobile phase (*e.g*. pH) [8] or variation of the phase ratio [9] may all occur. These selectivity changes can be manipulated to optimise resolution (Eq. 4), which can be a more convenient approach than changing the mobile phase or column.

Increasing temperature should also have a positive benefit on H values at high linear velocities due to increased diffusion and improved mass transfer. Conversely, the B-term contribution to band broadening will be significantly increased due to larger diffusion coefficients; this latter effect depends on the molecular weight of the analyte and affects the performance if operating around the optimum of the curve and particularly at sub-optimum linear velocities. These effects are made apparent in the profile of H, u_0 plots as the influence of the C term is substantially reduced while the minimum of the curve is shifted to a higher linear velocity value. Furthermore, increased temperature results in a reduction in the viscosity of the mobile phase allowing a wider range of flow rates and column lengths to be employed for improved kinetic performance.

Other than benefits to kinetic performance, resolution can also be controlled by temperature induced changes in selectivity. Temperature variation can also be used analogously to variation of the mobile phase composition during the separation process (*i.e*. gradient elution) to extend the range of solutes amenable to separation in a single

analysis [10-11]. This can be advantageous in comparison to conventional gradient elution using capillary columns as it eliminates the delay volume problem [12].

4. Column chemistry and morphology

4.1. Mechanisms of interaction

To effect a chromatographic separation, the solutes of interest must exhibit some affinity for both the stationary and mobile phase environments. There is a wide range of chemical properties that can be exploited for this purpose including hydrophobicity, ionic charge, molecular size, entropic effects and chirality. Two of the most commonly employed mechanisms for HPLC are *reversed-phase* and *ion-exchange* techniques.

The term reversed-phase was derived to contrast from the terminology applied to early HPLC methods which used unmodified silica or alumina stationary phases with a strong affinity for polar compounds (termed *normal-phase*). In reversed-phase HPLC retention is dependent upon the hydrophobic properties of compounds and the mobile phase is typically an aqueous-organic mixture (*e.g*. water-methanol) with a hydrophobic stationary phase (*e.g*. octadecylsilane). As this environment promotes partitioning of the solute based on its hydrophobicity, reversed-phase HPLC is applicable to a wide range of small to large compounds with low polarity. Reversed-phase columns are the most commonly used for HPLC applications from small molecules to large biomolecules such as intact proteins.

Coulombic interactions provide a convenient method for separation of compounds according to their charge using the principles of ion-exchange (IEX). For this type of analysis, the stationary phase contains chemically bonded ion-exchange sites with *fixed ions* and oppositely charged *counter ions*. For example, for the analysis of anions the stationary phase must contain fixed cationic groups to interact with anionic solutes to

retain them (anion-exchange). While the separation of cations requires a stationary phase containing fixed anionic groups with which cationic solutes can interact (cation-exchange). The mobile phase is comprised of a so-called *competing ion* which drives the equilibrium such that a single monovalent ion displaces a single monovalent counter ion to maintain electroneutrality [13]. Ion-exchange chromatography is frequently applied for analysis of small inorganic and organic ions and also for protein purification.

4.2. Packed columns

Currently the most common column format for HPLC, packed columns are prepared by tightly packing (normally spherical) particles of stationary phase material in a column with frits in place to hold the packed bed in place (Figure 3a). This type of column has a long history of development in reduction of the particle size, modification of chemical functionality, changing the porous properties and improving the regularity of particles and packing structure [14].

Packed columns can be prepared by a number of packing techniques including dry-packing, high pressure slurry packing and supercritical fluid (CO_2) packing [15]. Generally, for particle diameters of less than 20 μm, the high-pressure slurry technique is most commonly used [16]. This involves preparation of a dispersion of particles in a liquid medium that is filled into a reservoir and a high pressure (100-400 bar) applied to pack the particles into a cylindrical column. Numerous types of materials are used for particulate column packings including silica, polymer, zirconia, graphitic carbon and diamond. One important requirement for any packed column is the use of retaining frits at the termini of the column to hold all of the stationary phase particles in the column. In the case of capillary columns (< 0.5 mm internal diameter) this is commonly prepared by heating a

small amount of silica particles inside the column terminus to produce a porous, fused silica frit.

Packed columns remain the performance benchmark for most modes of HPLC. For example, a state of the art 50 cm long capillary column packed with porous sub-2 μm particles can achieve 200,000 theoretical plates for early eluting compounds operating close to the optimum linear velocity applying 1,600 bar in the reversed-phase mode [17]. This excellent performance improvement over early HPLC columns has been due to the extensive theoretical and practical advances in development of small (<3 μm) spherical particles with a narrow size distribution and porous properties tailored to suit diffusive mass transport [17-18].

4.3. Monolithic columns

The term “monolith” in this context refers to a stationary phase format that completely fills the column volume as a 'single particle' of porous material (Figure 3b). This type of stationary phase is characterised by a macroporous, continuous structure typically made from organic polymers [19] or silica precursors [20]. Monolithic columns for HPLC are typically prepared *in situ* from a mixture of precursors and solvents.

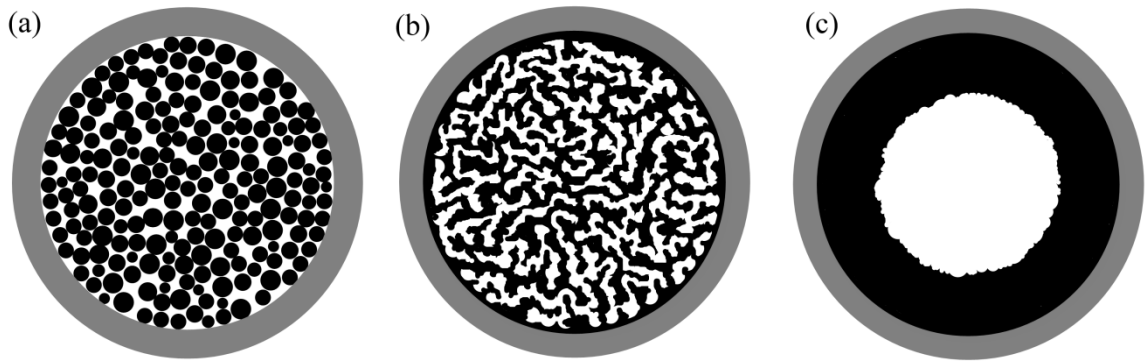

Figure 3. Schematic representation of column formats: (a) packed, (b) monolithic and (c) open-tubular.

The preparation of silica monoliths utilises a sol-gel transition involving the hydrolysis and polycondensation of alkoxides in an alcohol/water/silica system. An understanding of the two known modes of phase separation in this system (nucleation and growth and spinodal decomposition) allows control of the phase separation onset to yield fine or coarse structures without alteration of the gelling/freezing process. This allows all of the pore characteristics to be controlled by adjustment of the starting composition in an isothermally polymerising system [21]. Typical silica monoliths for HPLC are a silica skeleton with a bimodal pore structure of macropores (~1-3 μm) and mesopores (~0.01 μm) and have been most successfully utilised for small molecule separations [20,22].

Polymeric monoliths are prepared from a mixture of organic monomers, porogenic solvents and an initiator. The polymerisation can be initiated by a number of methods, but is typically performed using heat or irradiation. The pore size can be controlled to a certain extent by variation of the type and concentration of monomers, crosslinking agents and porogenic solvents [23]. As a large number of organic monomer precursors exist, there is a wide range of morphological and chemical properties that can be obtained using this approach.

One of the major goals in development of monolithic columns is the increased permeability with respect to packed columns. Coupled with a suitable pore structure, monolithic columns can be made longer and operated at higher linear velocities than packed columns allowing access to improved kinetic performance. Research on new methods for the optimisation of the morphology and porosity has allowed modern silica monoliths to yield improved kinetic performance over conventional packed columns [24]. However, this is currently only possibly for demanding separations requiring large plate numbers (*e.g.* N >50,000) with long columns (L >30 cm) [25].

Improving the chromatographic performance of polymer-based monolithic columns is currently more challenging as efficiency is generally poor despite the oft-cited benefits of convective driven mass transfer. Some recent efforts in improving the performance for separations of small molecules has had some success [26-27], but most applications for polymer monoliths demonstrated are for biomolecule separations performed in gradient elution mode where the structure and chemistry of the polymer limits non-specific adsorption in comparison to silica based materials. Notwithstanding, polymer monoliths offer several practical advantages as chromatographic supports including the wide range of available organic monomers, structural integrity, low flow resistance and stability under extreme pH and temperature conditions [28-30].

4.4. Open-tubular columns

Open-tubular columns are characterised by a film of stationary phase coating the surface of the column wall with a large flow-through region (Figure 3c). Despite their unquestioned dominance in capillary gas chromatography (GC), the practical implementations of open-tubular columns as HPLC columns is known to be hindered by the decreased diffusion in liquids (thus requiring narrower column diameters) and limited mass loadability (thus requiring thicker stationary phase coatings) [5,31].

There are a number of approaches for preparing open-tubular columns described in the literature. Most of these pertain to the use of thin, permanent coatings for application in capillary electrochromatography (CEC) [32]. Conversely, columns prepared for use in pressure driven mode typically have thicker films and are often prepared using a polymerisation step akin to those used for typical monolithic columns [33-34]. In this type of preparation, some control of the layer thickness can be achieved by adjusting the ratio of precursor(s) to solvent(s) with respect to the capillary diameter.

Early practical investigations into thin-film open tubular columns [35-38] and theoretical studies [31,39-42] indicated that improvements over packed bed columns can only be obtained when the total column diameter was approximately two times the particle size or less [31]. As such, very narrow column diameters (1-10 μm i.d.) are required, which poses problems due to limitations of detection and mass loadability. However, increasing the thickness of the film shifts the optimum column diameter to larger and more practical values, reducing some of the problems of column manufacturing. The use of thick-films can thus be used to yield trade-off between chromatographic performance and mass loadability [42].

5. Scope of thesis

This thesis primarily focuses on application of theoretical and practical performance optimisation strategies for a range of HPLC approaches. The work has a strong focus on optimisation of the theoretical plate number and kinetic performance (*i.e.* efficiency requirements) for a variety of chromatographic modes and applications. A secondary focus of the work was improving chromatographic resolution utilising variation of temperature.

Chapter I focuses on the principles and preparation of kinetic plots to characterise the performance in isocratic and gradient LC. This graphical approach allows the selection of columns (*i.e.* optimum particle size and column length) and LC conditions (operating pressure and temperature) to generate a specific number of plates or peak capacity in the shortest possible analysis time. Instrument aspects including the influence of extra-column effects (maximum allowable system volume) and thermal operating conditions (oven type) on performance are discussed. In addition, the performance characteristics of porous-shell particle packed columns and monolithic stationary phases are presented and the potential of future column designs is discussed.

In **Chapter II**, the performance of alternative approaches to conventional acetonitrile gradient methods for reversed-phase liquid chromatographic analysis of intact proteins were investigated using commercial poly(styrene-*co*-divinylbenzene) monolithic columns. Alternative solvents to acetonitrile (2-propanol and methanol) coupled with elevated temperatures demonstrated complementary approaches to adjusting separation selectivity and reducing organic solvent consumption. Only minor (<5%) decreases in detectable protein recovery occurred between 40-100°C on the timescale of separation (2-5 minutes), while reducing the viscosity of a 2-propanol/water eluent by elevating the separation temperature permitted serial coupling of three columns to increase peak production. Narrow-bore (1 mm i.d.) columns were also found to provide a more suitable avenue to fast, high temperature (up to 140°C) separations.

A more detailed kinetic performance appraisal of organic polymeric monolithic columns also using commercial poly(styrene-*co*-divinylbenzene) monolithic columns was investigated in **Chapter III**. Analysis of a protein digest sample at elevated temperatures (≥80°C) indicated no apparent solute degradation, but comparison between low molecular weight solute and peptide separations highlighted the markedly different mass transport processes observed on macroporous monolithic beds and an improved *C* term at elevated temperature in both instances. The current usefulness of this column format for biomolecule analysis was further studied using a kinetic performance characterisation for the first time to provide direction for column development servicing this application.

In **Chapter IV** an investigation of the performance of polymer-based particulate columns for ion-exchange separations of small inorganic anions using a kinetic plot characterisation is presented. The influence of solute type, particle size, temperature and maximum pressure drop upon theoretical extrapolations was investigated with data collected using anion-exchange polymeric particulate columns. The quality of extrapolations was found to

depend upon the choice of solute, but could be verified by coupling a series of columns. Separations of small anions yielding 25,000-40,000 theoretical plates using five serially connected columns (9 μm particles) were obtained and yielded deviations of <15% from predictions. While this approach for achieving high efficiencies results in a very long analysis time (t_0 = 21 min), separations yielding approximately 10,000 theoretical plates using two serially connected columns (t_0 < 5 min) were shown to be more practically useful for isocratic separations when compared to use of a single column operated at optimum linear velocity (t_0 > 10 min).

This approach was extended using a novel gradient kinetic plot method for the performance characterisation of gradient separations of small inorganic anions in **Chapter V**. The method employed requires only information obtained from a series of isocratic column performance measurements and *in silico* predictions of retention time and peak width under gradient conditions. Results obtained under practically constrained conditions allow some solutions to generation of high peak capacities and rapid peak production for fast analysis to be determined. Using this prediction method, a maximum theoretical peak capacity of 84 could achieve separation of 26 components using a 120 minute gradient (R_s > 1). This approach provides a highly convenient tool for development of both mono- and multidimensional LC methodologies as it yields comprehensive understanding of the influence of gradient slope, analysis time, column length and temperature upon kinetically optimised gradient performance.

In **Chapter VI**, the optimisation of open-tubular column design for LC was investigated using a theoretical approach. To alleviate the low mass loadability and a corresponding poor concentration detectability of open-tubular liquid chromatography (OTLC) columns, much effort has been directed toward increasing the surface area and thickness of coatings. The kinetic optimisation of this column format was revisited by taking all thick-film

effects for porous coatings on retention, column resistance, band broadening and mass loadability into account. Considering the most advantageous case (*i.e*. where the retentive layer allows for the same high internal diffusion coefficient as conventional porous particles), calculations show the need for the development of coating procedures leading to porous films filling up approximately 50 to 70% of the total column diameter. Some predictions of practical targets for OTLC column development are presented including the use of elevated temperatures to increase the allowable column diameter.

Chapter VII explores the use of dynamic temperature changes on chromatographic resolution for ion-exchange separations of small anions. Thermoelectric heating of capillary columns enables accurate temperature gradient programming and rapid temperature pulses during the separation process. This approach has some advantages over solvent gradient elution methods as the rapid change of temperature can enable improved resolution between two peaks of interest without substantially influencing the rest of the separation.

6. Future work

Additional research that can follow the work in this thesis can be considered in three categories: firstly to improve the chromatographic performance of the organic polymer based stationary phases studied in **Chapters III-V**; secondly to develop new, thick-film open-tubular columns as guided by the theoretical work from **Chapter VI**; and finally to develop a better fundamental understanding of temperature changes for manipulating retention and selectivity in HPLC as experimentally shown in **Chapters II & VII**.

Theoretical direction for improving the performance of monolithic supports is provided from a combination of detailed experiment [43-44], computational fluid dynamics [45-46] and an understanding of kinetic performance characterisation [6]. The overwhelming

conclusion of these studies (and supported by experiment in **Chapter III**) is that currently available monoliths generally suffer from a broad pore-size distribution and poor bed homogeneity. Simply put, monoliths with a reduced domain size and improved bed homogeneity will benefit most from the enhanced permeability of this column format by virtue of reduced eddy dispersion [46]. Some attention has been paid to polymeric monoliths recently with research on shortened polymerisation times [26], variation of polymerisation conditions [47-48] and secondary hypercrosslinking [27]. While these approaches provide some performance improvement (although none have characterised kinetic performance) these columns still lag far behind silica monoliths and particulate columns given the minimum plate heights (H_{min}) are still >20 μm for retained compounds.

This ostensibly leads to two important research goals: 1) improving the structural homogeneity of polymer monoliths and 2) demonstrating applications where these columns provide a clear advantage over alternative technologies. In the case of the former, the task is challenging as it may require novel polymerisation methods using templates [49-50] or silica-polymer hybrids [51] to improve the structural homogeneity and reduce the contribution of eddy dispersion to band broadening. In order to obtain higher efficiencies with monolithic stationary phase technology, new highly ordered structures may be needed and consequently novel polymerisation methods (which sometimes are found in other fields not related to separation science) are required. In this regard, it is also important to consider that quantification of performance improvement for monolithic columns requires measurement of both the efficiency (*via* the conventional van Deemter approach) and the bed permeability to allow an unbiased estimate of the optimum column performance [6]. In doing so, this avoids erroneous over-estimation of column performance by use of measures such as plates/m as practical column lengths and analysis times can be properly considered.

The polymer-based ion-exchange stationary phases studied in **Chapters IV & V** can benefit greatly in terms of kinetic performance by decreasing the particle size. Unlike the polymer monoliths, the regularity of packed beds of polymer particles is more easily optimised with good packing procedures and a narrow particle size distribution. Using smaller particles may in turn require higher backpressures to be employed, but as shown by development of small particles for HPLC using reversed-phase materials significant performance improvements are achievable. One difficulty that may be encountered for this approach is the synthesis of very small polymeric particles with ion-exchange functionality.

The development of new thick-film open tubular columns discussed in **Chapter VI** is a challenging task, but one that can offer great performance if technical challenges can be overcome. Aiming for 5 μm i.d. columns should be an achievable goal if using sensitive mass spectrometric detection. The major obstacles to realising this possibility are the difficulties in manufacturing 1-2 m long columns reproducibly and the instrumental constraints associated with nanolitre scale volumes and detection limits. Notwithstanding these difficulties, some practical development in this area can be achieved particularly if gradient elution mode is employed.

The stationary phase morphology and chemistry play an important role in the future of high temperature HPLC (HTLC) as the stability of many formats is questionable under high temperature conditions. Polymer monolith columns have been shown to have excellent thermal stability under HTLC conditions in **Chapter II** and provide an ideal platform for research in this field. From an instrumental perspective, **Chapter VII** illustrates that practical applications of HTLC continue to show most promise using capillary column formats and novel heating devices with thermoelectric elements [52-53]. Despite the lesser effect of temperature on selectivity and retention compared to solvent

strength, the ability to rapidly vary temperature is complementary to that offered by changing the solvent gradient composition as temperature alteration can be transient or confined to one section of the chromatographic column.

The modelling of heat transfer properties in a low thermal mass HTLC system by Verstraeten *et al*. [54] illustrates the great potential of narrow bore (<1 mm i.d.) columns in conjunction with relatively fast heating and cooling rates. Thus, future work in this area should also be focused on developing strategies for temperature profile optimisation akin to the separation simulation approaches developed for solvent gradient elution profiles. In this regard, applications for ionisable solutes are particularly amenable to this approach as the retention behaviour is often vastly different between solutes contained in typical samples (*e.g*. exothermic vs. endothermic) and temperature can play a vital role in resolution optimisation.

7. References

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I

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Review Article

Kinetic performance optimisation for liquid chromatography: Principles and practice

This HPLC tutorial focuses on the preparation and use of kinetic plots to characterise the performance in isocratic and gradient LC. This graphical approach allows the selection of columns (i.e. optimum particle size and column length) and LC conditions (operating pressure and temperature) to generate a specific number of plates or peak capacity in the shortest possible analysis time. Instrument aspects including the influence of extra-column effects (maximum allowable system volume) and thermal operating conditions (oven type) on performance are discussed. In addition, the performance characteristics of porous-shell particle-packed columns and monolithic stationary phases are presented and the potential of future column designs is discussed.

Keywords: High temperature / Kinetic plot / Monolith / Poppe plot / Ultra high-pressure liquid chromatography
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II

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High temperature liquid chromatography of intact proteins using organic polymer monoliths and alternative solvent systems

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ABSTRACT

Alternative approaches to conventional acetonitrile gradient methods for reversed-phase liquid chromatographic analysis of intact proteins have been investigated using commercial poly(styrene-*co*-divinylbenzene) monolithic columns (Dionex ProSwift™ RP-2H and RP-4H). Alternative solvents to acetonitrile (2-propanol and methanol) coupled with elevated temperatures demonstrated complementary approaches to adjusting separation selectivity and reducing organic solvent consumption. Measurements of peak area at increasing isothermal temperature intervals indicated that only minor (<5%) decreases in detectable protein recovery occurred between 40 and 100 °C on the timescale of separation (2–5 min). The reduced viscosity of a 2-propanol/water eluent at elevated temperatures permitted coupling of three columns to increase peak production (peaks/min) by 16.5%. Finally, narrow-bore (1 mm i.d.) columns were found to provide a more suitable avenue to fast, high temperature (up to 140 °C) separations.

III

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Kinetic performance appraisal of poly(styrene-*co*-divinylbenzene) monolithic high-performance liquid chromatography columns for biomolecule analysis

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ABSTRACT

A broad appraisal of the kinetic performance of organic polymeric monolithic columns is reported using commercially available poly(styrene-*co*-divinylbenzene) monolithic columns (Dionex ProSwift™ RP-1S). Analysis of a protein digest sample at elevated temperatures (≥80 °C) indicated no apparent analyte degradation using an inert polymeric stationary phase. Comparison between low molecular weight solute and peptide separations highlighted the markedly different mass transport processes observed on macroporous monolithic beds and an improved *C* term at elevated temperature in both instances. The current usefulness of this column format for biomolecule analysis was further studied via employment of a kinetic performance characterisation for the first time to provide direction for column development servicing this application.

IV

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Probing the kinetic performance limits for ion chromatography. I. Isocratic conditions for small ions

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ABSTRACT

The first use of the kinetic plot method to characterise the performance of ion-exchange columns for separations of small inorganic anions is reported. The influence of analyte type (mono- and divalent), particle size (5 and 9 μm), temperature (30 and 60 °C) and maximum pressure drop upon theoretical extrapolations was investigated using data collected from anion-exchange polymeric particulate columns. The quality of extrapolations was found to depend upon the choice of analyte, but could be verified by coupling a series of columns to demonstrate some practical solutions for ion chromatography separations requiring relatively high efficiency. Separations of small anions yielding 25–40,000 theoretical plates using five serially connected columns (9 μm particles) were obtained and yielded deviations of <15% from the kinetic plot predictions. While this approach for achieving high efficiencies results in a very long analysis time (t_0 = 21 min), separations yielding approximately 10,000 theoretical plates using two serially connected columns (t_0 < 5 min) were shown to be more practically useful for isocratic separations when compared to use of a single column operated at optimum linear velocity (t_0 > 10 min).

V

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Probing the kinetic performance limits for ion chromatography. II. Gradient conditions for small ions

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ABSTRACT

A gradient kinetic plot method is used for theoretical characterisation of the performance of polymeric particulate anion exchange columns for gradient separations of small inorganic anions. The method employed requires only information obtained from a series of isocratic column performance measurements and *in silico* predictions of retention time and peak width under gradient conditions. Results obtained under practically constrained conditions provide parameters for the generation of high peak capacities and rapid peak production for fast analysis to be determined. Using this prediction method, a maximum theoretical peak capacity of 84 could be used to achieve separation of 26 components using a 120 min gradient ($R_s > 1$). This approach provides a highly convenient tool for development of both mono- and multidimensional ion chromatography (IC) methodologies as it yields comprehensive understanding of the influence of gradient slope, analysis time, column length and temperature upon kinetically optimised gradient performance.

VI

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Kinetic optimisation of open-tubular liquid-chromatography capillaries coated with thick porous layers for increased loadability

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ABSTRACT

The kinetic optimisation of open-tubular liquid chromatography (OTLC) columns has been revisited by taking the thick-film effects for porous coatings on retention, column resistance, band broadening and mass loadability into account. Considering the most advantageous case (*i.e.* where the retentive layer allows for the same high internal diffusion coefficient as conventional porous particles), calculations show the need for the development of coating procedures leading to porous films filling up approximately 50–70% of the total column diameter. Furthermore, to achieve optimum kinetic performance for separations of small molecules with total analysis times of less than 8 h ($k' = 9$), total column diameters should be less than 6 μm with lengths typically greater than 0.8 m for N values of 125,000–500,000 at a pressure of 400 bar. The use of elevated temperature LC (90 °C) is also shown to increase the allowable total column diameter to up to 9 μm for a larger range of N values (100,000–880,000).

VII

analytical chemistry

Article

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Temperature Pulsing for Controlling Chromatographic Resolution in Capillary Liquid Chromatography

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Supporting Information

ABSTRACT: In this study we introduce the implementation of rapid temperature pulses for selectivity tuning in capillary liquid chromatography. Short temperature pulses improved resolution in discrete sections of chromatograms, demonstrated for ion-exchange chromatography (IC) and hydrophilic interaction chromatography (HILIC) modes. Using a resistively heated column module capable of accurate and rapid temperature changes, this concept is first illustrated with separations of small anions by IC using a packed capillary column as well as a series of nucleobases and nucleosides by HILIC using a silica monolithic column with zwitterionic functionality (ZIC-HILIC). Both positive (increasing temperature) and negative temperature pulses are demonstrated to produce significant changes in selectivity and are useful approaches for improving resolution between coeluted compounds. The approach was shown to be reproducible over a large number of replicates. Finally, the use of temperature gradients as well as other complex temperature profiles was also examined for both IC and HILIC separations.